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Günter Mayer, Christina Lünse, Colin Suckling and Fraser Scott

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Novel TPP-riboswitch activators bypass metabolic enzyme dependency

Christina E. Lünse[1], Fraser J. Scott[2], Colin J. Suckling[2,*], and Günter Mayer[1,*]

[1]Life and Medical Sciences Institute, University of Bonn, Bonn, Germany
[2]Department of Pure & Applied Chemistry, University of Strathclyde, Glasgow, United Kingdom

*** Correspondence:** Günter Mayer, gmayer@uni-bnn.de, c.j.suckling@strath.ac.uk

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Abstract

Riboswitches are conserved regions within mRNA molecules that bind specific metabolites and regulate gene expression. TPP-riboswitches, which respond to thiamine pyrophosphate (TPP), are involved in the regulation of thiamine metabolism in numerous bacteria. As these regulatory RNAs are often modulating essential biosynthesis pathways they have become increasingly interesting as promising antibacterial targets. Here, we describe thiamine analogs containing a central 1,2,3-triazole group to induce repression of *thiM*-riboswitch dependent gene expression in different *E. coli* strains. Additionally, we show that compound activation is dependent on proteins involved in the metabolic pathways of thiamine uptake and synthesis. The most promising molecule, triazolethiamine (TT), shows concentration dependent reporter gene repression that is dependent on the presence of thiamine kinase ThiK, whereas the effect of pyrithiamine (PT), a known TPP-riboswitch modulator, is ThiK independent. We further show that this dependence can be bypassed by triazolethiamine-derivatives that bear phosphate-mimicking moieties. As triazolethiamine reveals superior activity compared to pyrithiamine, it represents a very promising starting point for developing novel antibacterial compounds that target TPP-riboswitches. Riboswitch-targeting compounds engage diverse endogenous mechanisms to attain *in vivo* activity. These findings are of importance for the understanding of compounds that require metabolic activation to achieve effective riboswitch modulation and they enable the design of novel compound generations that are independent of endogenous activation mechanisms.

Introduction

Riboswitches are RNA elements mostly found in the 5' UTR of bacterial mRNA that specifically sense the concentration of a small metabolite. Upon metabolite binding, substantial conformational changes occur that ultimately result in an on- or off-switch of gene expression. These regulatory elements represent a fundamental new means of controlling cellular processes in response to environmental conditions (Breaker 2011). Riboswitches often regulate expression of essential genes and as such they are interesting target structures for the development of novel antibiotic compounds (Blount and Breaker 2006). Artificial compounds acting successfully on riboswitches have to meet at least two criteria: i) they must specifically bind to the relevant RNA structure and ii) induce the conformational changes that finally lead to down- or up-regulation of gene expression. In recent years synthetic molecules that meet these criteria have been discovered for several riboswitch classes (Lünse, Schüller et al. 2014). One of them is the TPP-riboswitch (Miranda-Rios, Navarro et al. 2001; Winkler, Nahvi et al. 2002), which selectively interacts with thiamine pyrophosphate (TPP) by binding to its pyrimidine moiety through the so-called pyrimidine sensor helix (P2, J2-3, P3) of the aptamer domain. Conformational changes of the riboswitch resulting in repression of gene expression are only achieved if the pyrophosphate group of the ligand is recognized by the pyrophosphate sensor helix (P4, J4-5, P5, Figure 1A) (Edwards and Ferre-D'Amare 2006; Serganov, Polonskaia et al. 2006; Thore, Leibundgut et al. 2006). While this riboswitch class has been shown to allow a greater degree of variation at the position of the thiazole ring, exemplified by the thiamine analog pyrithiamine (PT, Figure 2A), the presence of the pyrophosphate moiety is mandatory to interact with and, more importantly, to induce switching of the *E. coli thiM*-riboswitch (Winkler, Nahvi et al. 2002; Rentmeister, Mayer et al. 2007). This necessity impedes the development of compounds that act on TPP-riboswitches, since either they cannot passively pass the cell wall due to the highly charged pyrophosphate moiety or they cannot efficiently activate the riboswitch if lacking this group. PT's *in vivo* activity most likely relies on hijacking endogenous metabolic enzymes that phosphorylate exogenously added PT, thereby yielding the active derivative pyrithiamine pyrophosphate (PTPP) (Iwashima, Wakabayashi et al. 1976). In this report we elaborate on a series of triazolethiamines (TT) that have been shown recently to act on TPP-riboswitches *in vitro*, if diphosphorylated yielding triazolethiamine pyrophosphate (TTPP). We investigated TT-activity in relation to the length and modification of the alkyl chain and the presence of endogenous proteins involved in thiamine metabolism. We found that the activity of triazolethiamine (TT) depends on the metabolic enzyme thiamine kinase (ThiK) and thiamine transporters. Most importantly, we demonstrated that ThiK dependency is bypassed by triazolethiamine-derivatives that bear phosphate mimicking and metal-ion chelating moieties. This strategy opens new avenues towards riboswitch activating compounds, in particular those that rely on phosphate groups to recognize and switch cognate RNA structures.

Materials and methods

β-Galactosidase reporter gene assay

For β-galactosidase assays 5 ml pre-cultures of DH5αZ1, BW25113 or Keio deletion strains containing the appropriate plasmid constructs were prepared in LB Lennox standard medium

(lysogeny broth Lennox, 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl in water) and incubated over night at 37°C and 155 rpm. Incubation was followed by photometric measurement of optical density at λ= 600 nm (OD_{600}) and dilution of cells to an optical density of 0.5. This dilution was used to inoculate β-galactosidase expression cultures at a ratio of 1:500 in a final volume of 2 or 4 ml in M9 medium (5x M9 medium containing 15 g/l KH_2PO_4, 5 g/l NH_4Cl, 2.5 g/l NaCl, 30 g/l Na_2HPO_4) containing final concentrations of 5 mM $MgSO_4$, 0.2 wt% glucose, 0.2 µg/µl casamino acids (Difco), and 100µg/ml ampicillin. Even though vitamin-deprived casamino acids were used, growth of thiamine auxotrophic strains was observed without addition of thiamine indicating that a small, but constant amount of thiamine must be present in the casamino acid stock. Minimal inhibitory concentration determinations in casamino acid free minimal medium with separately added amino acids revealed that at least 1 nM of thiamine is necessary to enable bacterial growth of the thiamine auxotrophs investigated. The expression cultures contained either 20 or 500 µM of thiamine (Sigma), 500 µM pyrithiamine (Sigma) or 500µM of the compounds, respectively. Controls received neither thiamine, or pyrithiamine nor compounds. If compounds had to be dissolved in DMSO, controls were supplemented with equal amounts (final DMSO concentration: 1%). The cultures were incubated for 24 h at 37°C and 150 rpm. After 24 h incubation the optical density was measured. The cells were centrifuged at 4500 g for 5 min and the pellets were washed twice in 400 µl 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4x2H_2O$, 2 mM KH_2PO_4, pH 7.4). The pellets were resuspended in 200 µl 1x lysis buffer (Reporter Lysis Buffer, Promega), incubated for 15 min at room temperature and cells were pelleted. Supernatants were used for all following steps. 75 µl of the respective lysates were mixed with 75 µl of 2x assay buffer (200 mM sodium phosphate buffer pH 7.3, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, 1.33 mg/ml ONPG) and incubated for 5-15 min at 37°C. The reaction was stopped by the addition of 250µl 1 M Na_2CO_3 and absorbance at 420 nm was measured immediately using a Nanoquant (Infinite 200, Tecan). Reporter gene assays were performed in clear, flat bottom 96-well plates. Miller units were calculated according to the following formula: Miller units = (1000*OD420)/(OD 600*incubation time [min]*culture volume [L]) and Miller units of controls without thiamine or compound added were set to 1. Samples were run in duplicate and experiments were repeated at least three times.

Results

Thiamine analogs that bear a 1,2,3-triazole group instead of the thiazole heterocyclic moiety as found in thiamine were synthesized and investigated for TPP-riboswitch activation using a β-galactosidase reporter gene assay (Figure 1B). In this assay the TPP-riboswitch *thiM* from *E. coli* was cloned into the 5´-UTR of β-galactosidase. The DH5αZ1 *E. coli* strain was transformed by this plasmid (Simons, Houman et al. 1987; Diederich, Rasmussen et al. 1992; Lutz and Bujard 1997). Due to the thi-1 mutation, DH5αZ1 cells are thiamine auxotroph, which means they are dependent on thiamine uptake from the growth medium (Supplementary Table 1). This enables direct external control of the availability of this vitamin. Synthesis of TT-compounds was achieved using a ‘click’ chemistry approach utilizing a common azide intermediate, which was then further reacted with the appropriate substituted alkyne yielding corresponding thiamine analogs (Figure 2A, Supplementary Figure 1). Subsequent monitoring of β-galactosidase activity was performed in the presence and absence of thiamine or triazolethiamine (TT) derivatives.

Addition of thiamine to the *E. coli* cultures resulted in a complete loss of β-galactosidase expression; an IC_{50}-value of 0.015 µM was observed (Figure 2 and Table 1). In line with previous findings, pyrithiamine (PT) was shown incapable of β-galactosidase repression in DH5αZ1 (Figure 2B)(Sudarsan, Cohen-Chalamish et al. 2005). On the other hand, the addition of 500µM TT (**1**) represses the expression of β–galactosidase expression to a level similar to that of thiamine. TT has been shown to depend on diphosphorylation to gain activity *in vitro* (Chen, Cressina et al. 2012). Thus, we anticipate that TT is phosphorylated by the endogenous set of thiamine converting enzymes. Shortening (**2**) or elongating the alkyl side chain (**3** and **4**, Figure 1a) results in a loss of repression with increasing deviation from the natural alkyl chain length (Figure 1B). This is most likely due to reduced uptake and/or phosphorylation of these compounds, but could also be because of a poor fit of phosphorylated triazolethiamines to the riboswitch. Previously, it has been shown that the *thi*-box aptamer domain is somewhat adjustable to ligand size through compaction of pyrimidine and phosphate sensor helices (Edwards and Ferre-D'Amare 2006). However, this low affinity ligand binding (thiamine K_D= 50µM vs. TPP K_D= 50nM, (Sudarsan, Cohen-Chalamish et al. 2005)) is unlikely to efficiently induce subsequent changes in the expression platform (Serganov, Polonskaia et al. 2006) and thus reporter gene expression. Interestingly, compound **5**, which bears an amino group instead of a hydroxyl residue on the ethyl-alkyl side chain of TT, also represses reporter gene activity. How this compound would be activated through *in vivo* phosphorylation is unclear. However, it is possible that **5** is also phosphorylated in the same way as TT. Experiments using a mutant version of the *thiM* riboswitch (*thiM*-Mu, Figure 1A), which is incapable of binding to TPP showed that neither of the investigated TT-compounds represses gene expression in these assays. These data underline that repression of gene expression truly relies on riboswitch binding and activation. We next measured IC_{50}-values of these compounds, to evaluate concentration dependent repression of gene expression. TT (**1**) revealed the strongest effect with an IC_{50}-value of 8.4 µM (Table 1 and Supplementary Figure 2). Compounds with increasing alkyl-chain length (**3** and **4**) showed no concentration dependent inhibition curves (Table 1) and, as expected for this bacterial strain, PT did not show an effect on β-galactosidase expression (Table 1, Figure 2A). In order to investigate the dependence of **TT** activity on endogenous proteins and enzymes involved in thiamine biosynthesis, several strains of the Keio collection were investigated, that all originate from the strain BW25113, and contain deletions of non-essential genes known to be involved in thiamine metabolism of *E. coli* (Scheme 1) (Baba, Ara et al. 2006).

Thiamine is synthesized from two precursors, hydroxymethylpyrimidine diphosphate (HMP-PP) and hydroxyethylthiazole phosphate (HET-P), which are produced independently and finally joined to form thiamine phosphate (Begley, Downs et al. 1999). Further phosphorylation yields the cofactor TPP (Scheme 1). The Keio deletion strains were transformed with the β-galactosidase reporter plasmid containing either the wild type or the mutant TPP-riboswitch and effects of thiamine, **TT** or **PT** on reporter gene expression were assayed (Table 2, Figure 3, Supplementary Figures 9 and 10). In most strains the addition of thiamine led to a strong decrease in β-galactosidase expression that is comparable to that observed for the wild type strain. As expected, the *thiK* deletion strain (*ΔthiK*) revealed decreased thiamine sensitivity, since this enzyme is required for the phosphorylation of thiamine to TPP. When adding thiamine, *thiI* and *iscS* deletion strains showed slightly increased levels of reporter gene expression indicating a loss of TPP-riboswitch activation (Figure 3A). Investigating the impact of **TT** (Figure 3B) on TPP-

riboswitch dependent reporter gene expression in the deletion strains revealed a prominent increase in reporter gene expression for those strains containing deletions of thiamine transport genes (Scheme 2; *thiP*, *thiQ*, *tbpA*) in comparison to wild type cells (Figure 3B, magenta bars). This may indicate that **TT** uptake is, at least in part, mediated by these ABC-transporters. However in the presence of **PT**, reporter gene expression in these strains was scarcely increased in comparison to the wild type strain (Figure 3C). This suggests that **PT**, just like thiamine (Figure 3A), must use other routes of cell entry apart from the thiamine-specific active transporter. Possibly, its positive charge supports passive diffusion processes, as believed to occur for thiamine as well (Webb, Claas et al. 1998). Besides the dependence on transport proteins, differential effects on several other deletion strains involved in phosphate-transfer or thiazole formation were observed (Figure 3, green and gray bars, respectively). Assaying *ΔthiK*, *ΔthiD* and *ΔthiM*, strains, whose deleted genes are involved in phosphate transfer reactions, revealed increased β-galactosidase expression in the presence of **TT** compared to wild type, whereas *ΔthiE* showed decreased β-galactosidase expression (Figure 3B). As the deletion of *thiE* renders the bacteria incapable of synthesizing any TPP from its HMP and HET precursors (Scheme 1), the reporter gene expression is mainly influenced by external thiamine or compound supply. Interestingly, the prominent decrease of β-galactosidase expression in the presence of **TT** is comparable to that found for thiamine at the same concentration in the wild type strain BW25113 (Figure 3A, black bars). In *ΔthiE* cells, lower intracellular concentrations of thiamine compete for any possible enzymatic **TT**-activation mechanism (such as phosphorylation or take-up). Therefore, the same amounts of **TT** lead to a stronger decrease of reporter gene expression in these cells in comparison to other deletion strains (Figure 3B). In the presence of **TT,** *thiC* and *iscS* deletion strains which are involved in formation of the thiazole moiety of thiamine revealed increased β-galactosidase expression (Figure 3B). At the same time β-galactosidase expression in *thiI*, *thiG*, *thiH* and *sufS* deletion strains remained unaffected or was slightly decreased in the presence of **PT** (Figure 3C). Finally, PT-treated *E. coli* strains containing deletions of the genes *thiD* and *thiC*, which are involved in the biosynthesis of the pyrimidine-containing precursor HMP-PP (Scheme 1), show an increase in reporter gene expression (Figure 3C). The same is observed for the *ΔthiE* strain. The deletion of *iscS* renders bacteria less susceptible to thiamine, **TT** or **PT** (Supplementary Figure 3). However, it should be noted that the *ΔiscS* strain showed slower growth than any of the other deletion strains investigated. This reduced fitness may be due to the fact that ThiI and IscS are not only involved in thiamine metabolism but also needed in the 4-thiouridine biosynthetic pathway (Scheme 1). Interestingly, deleting the thiamine kinase ThiK abolished thiamine and **TT** dependent inhibition of reporter gene expression (Figure 3A and 3B). This finding underlines the hypothesis that **TT** is only active *in vivo* in its phosphorylated form. Due to its close structural similarity to the natural substrate thiamine, **TT** is likely to be recognized and phosphorylated by endogenous bacterial enzymes such as ThiK. Using ITC measurements Chen and colleagues revealed that the chemically synthesized, diphosphorylated form of **TT**, named triazolethiamine pyrophosphate (TTPP), binds to the *thiM* riboswitch with an affinity of 370 nM, whereas for the natural ligand TPP a K_D of 8 nM was measured (Chen, Cressina et al. 2012). Additionally, *in vitro* translation assays proved that binding of TTPP induces a change in riboswitch secondary structure to sequester the SD-sequence and inhibits efficient protein translation (Chen, Cressina et al. 2012). Therefore, endogenous diphosphorylation of **TT** would generate a very potent TPP-riboswitch activator. Hence, we were intrigued to find that reporter gene expression for the *thiK* deletion strain is dramatically

decreased in the presence of **PT** (Figure 3C). This suggests that PT either does not need to be activated by pyrophosphorylation through ThiK, that it can also be phosphorylated by other endogenous enzymes like ThiE or that it is converted into TPP through salvage pathways.

In order to bypass ThiK-dependency of **TT** activity in *E. coli*, triazolethiamine derivatives that were intended to be independent of endogenous phosphorylation were synthesized and their impact on riboswitch regulation of β-galactosidase expression was studied. These compounds contain a number of phosphate mimics (sulfate, sulfonamide, and sulfone) and metal-chelating groups (dicarbonyl compounds) that might be expected to interact with phosphate-binding metal ions in proteins (Supplementary Figures 4-8). A 'click' chemistry approach was used to generate sulfone **7** and the methotrexate- or folate- like compounds **15** and **16**. Amide couplings were performed to obtain the sulfonamides **8** and **9** and also to obtain the pyruvate, malonate and salicylic acid containing compounds **10**, **11** and **12**, respectively (Erixon, Dabalos et al. 2008). Additionally, a Michael addition was used to generate the sulfones **13** and **14**.
With respect to the phosphate mimics, the sulfonamide **13** and the sulfone **14** that are connected to a benzyl group scarcely activate the *thiM* riboswitch in DH5αZ1 cells. The sulfonamide **8** had a similar effect on β-galactosidase expression in the thiamine auxotroph strain. Interestingly, compounds containing the planar benzene ring are not as active in *thiM* riboswitch activation, even though it is imaginable that this aromatic ring could tighten compound-riboswitch interaction by π-π-stacking with RNA nucleobases (Edwards and Ferre-D'Amare 2006; Thore, Leibundgut et al. 2006). Compounds **15** and **16** were found to be the most potent repressors of reporter gene expression (Figure 4B, ~0.5 fold β-galactosidase expression). Compounds of this group have enough flexibility in principle to interact with two metal ions via their ester or carboxyl groups. Interestingly, these molecules activate the *thiM* riboswitch extensively, despite their relatively large size. The carboxylate ethyl esters like **15** are likely to enter the cell passively and are probably hydrolyzed by esterases resulting in **16**. To investigate whether the most potent phosphate mimics exhibit a ThiK independent reduction in reporter gene expression, compounds **7** and **16** were chosen and assayed in the thiamine kinase deficient *E. coli* strain (Figure 5A). In contrast to addition of thiamine or **TT**, which do not reduce reporter gene expression, the addition of the same amount of **7** and **16** induces a decrease of β-galactosidase expression to approximately 0.25 fold (Figure 5A). However, *ΔthiK* strains transformed by the mutated *thiM* riboswitch construct do not or only slightly respond to thiamine or compound addition (Supplementary Figure 11A). In wild type *E. coli* strain BW25113 thiamine has the strongest influence on β-galactosidase repression. **TT** and compound **7** are almost as efficient in inhibiting gene expression whereas **PT** and compound **16** show somewhat weaker effects (Figure 5B). Again no significant influence on reporter gene expression in cells containing the mutant *thiM* riboswitch was observed (Supplementary Figure 11C). Investigating the deletion of one subunit of the active thiamine transporter (ThiP) revealed increased levels of reporter protein for **TT**, **7** and **16**, indicating that these molecules are at least in part taken-up via this mechanism (Figure 5C). The investigation of the mutant riboswitch construct *thiM*-Mu proves these compound effects to be specific for *thiM* riboswitch activation, as the mutated RNA does not alter protein expression (Supplementary Figure 11).

Discussion

TPP-box riboswitches are interesting and attractive target structures for developing antibacterial

compounds. They form the most extensive riboswitch class with representatives found in bacteria, archea and plants (Kubodera, Watanabe et al. 2003; Sudarsan, Barrick et al. 2003; Bocobza, Adato et al. 2007). Here we show that replacing the thiazole heterocycle with 1,2,3-triazole is a valuable strategy to generate thiamine analogs that interact with TPP-box riboswitches and, thus, induce repression of gene expression. These findings are in line with the observations of Chen al. recently reporting that the diphosphorylated triazolethiamine indeed interacts with the *thiM* riboswitch *in vitro*, albeit with decreased affinity (Chen, Cressina et al. 2012). We went a significant step further and demonstrated that these compounds are effective in *E. coli* and, more importantly, that their activity depends on proteins involved in the metabolic pathways of thiamine uptake and synthesis.

The data shown above indicate that activity of **TT** depends on active uptake and endogenous phosphorylation by ThiK. Surprisingly, **PT** was found to be independent of ThiK and transporters. Therefore, **PT** is either phosphorylated by other kinases apart from ThiK or its phosphorylation, against all previous hypotheses, is not necessary for *in vivo* activity (Edwards and Ferre-D'Amare 2006; Deigan Warner 2014). Unphosphorylated **PT** is known to have a more than 1000 fold lower affinity for the TPP-riboswitch, whereas diphosphorylation results in a K_D that is only 3fold higher than that of TPP (Sudarsan, Cohen-Chalamish et al. 2005). Although **PT** was shown to bind to the pyrimidine sensor helix of the aptamer domain by one hydrogen bond and the stacking of the aminopyrimidine ring, crystal structures revealed that two hydrogen bonds have been lost in comparison to TMP or TPP interactions. The pyrophosphate sensor helix is largely disordered and it is questionable whether this riboswitch conformation allows gene regulation upon **PT** binding (Edwards and Ferre-D'Amare 2006). Furthermore, the increase in *thiM*-riboswitch dependent reporter gene expression in the presence of **PT** in *ΔthiD* or *ΔthiC* strains suggests the existence of a **PT**-detoxification mechanism in *E. coli* as proposed for *B. subtilis* by Sudarsan et al. in 2005 (Sudarsan, Cohen-Chalamish et al. 2005). If indeed there was a thiaminase-II-like enzyme present in *E. coli*, HMP could be recovered from **PT** and used for the generation of thiamine through combination with *de novo* synthesized thiazole (Scheme 1). This **PT** recycling would lead to a decrease of its intracellular concentration, hence a diminished influence on the TPP-riboswitch-dependent gene expression. At the same time, the newly generated TPP is likely to be used immediately for its biological purpose without accumulation and hence a need for riboswitch-mediated down regulation of gene expression. Even though a TenA-like protein has not yet been identified in *E. coli*, *in silico* predictions indicate that ThiC may represent a functional equivalent of TenA (Morett, Korbel et al. 2003).

We further show that equipping **TT** with metal-chelating groups results in ThiK- and transport-independent activity. This indirectly proves that **TT** requires phosphorylation and indicates a possible route in riboswitch activator design which circumvents the necessity of endogenous proteins and renders riboswitch inhibitors independent of bacterial pathways. The most promising phosphate-mimics were tested for their dependence on ThiK or active thiamine transport mechanisms. Whereas activity of compound **16** appears to be ThiK-independent, its transport is strongly influenced by ABC transporter integrity. At the same time, effects of compound **16** in wild type cells are not as prominent as those observed for **TT**. Compound **7** however, does not only reveal strong reporter gene repression, but this effect is even independent of ThiK or ThiP presence. Hence, future compound designs should take characteristics of compound **7** into account in order to provide novel chemical entities whose *in vivo* activity does not depend on the individual enzyme repertoire of the target cell. Also, investigating potential routes of metabolism

of phosphate-mimicking compounds may unveil further information and support the generation of even more precise riboswitch-regulating compounds.

The distinct efficiencies of **TT**-derivatives and **PT** in repression of gene expression, further point at possible secondary effects of the employed metabolite analogs. These side effects may rely on interactions with enzymes involved in thiamine pyrophosphate metabolic pathways. Indeed it has been shown that triazole-derivatives of thiamine pyrophosphate inhibit enzymes involved in the biosynthesis of TPP *in vitro* (Erixon, Dabalos et al. 2007).

Others have shown that analogs with a positively charged middle ring bind much better to the *thiM* riboswitch than ones with a neutral ring at this position (Chen, Cressina et al. 2012). It was suggested that this is due to the electron drawing effect of the middle ring, which enhances the binding of the pyrimidine moiety to the cognate part of the aptamer domain. The reporter gene assay presented herein however suggests that *in vitro* binding does not always necessarily correlate with *in vivo* riboswitch regulation. Even though **TT** does not contain a charged middle ring, it induces reporter gene repression more efficiently than **PT**. Moreover, our study shows that riboswitch regulation by artificial activators is dependent on the individual set of metabolic enzymes present in the organism.

Since **TT (1)** is active in *E. coli* and reveals superior activity when compared to pyrithiamine it represents a very promising starting point for developing novel antibacterial compounds that target TPP-riboswitches. Furthermore, our study shows that even being very similar from a structural point of view, **TT** and **PT** engage diverse endogenous mechanisms to attain activity. These findings are of importance for the design and understanding of compounds that require intracellular activation to achieve an effective repression of gene expression.

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Table 1: IC_{50}-values of thiamine, pyrithiamine and compound **1-5** for inhibition of reporter gene expression in DH5αZ1 cells are listed. Means and 95% confidence intervals of at least three independent experiments, measured in duplicates, are shown. n. a. = not applicable; where data points could not be fitted to a dose-response curve. TT: Triazolethiamine; PT: Pyrithiamine

Compound	IC_{50} [µM] (DH5αZ1)	95% Confidence Interval
thiamine	0.015	0.012 to 0.019
1 (TT)	8.4	4.6 to 15.6
2	43.3	13.4 to 140
3	n. a.	n. a.
4	n. a.	n. a.
5	22.9	12.1 to 43.4
PT	n. a.	n. a.

Table 2: IC_{50}-values of thiamine for inhibition of reporter gene expression in *E. coli* containing the indicated deletion of a non-essential thiamine biosynthesis gene are shown as mean and 95% confidence interval of at least three independent experiments, measured in duplicates. n. a. = not applicable, where data points could not be fitted to a dose-response curve. Respective curves are shown in Supplementary Figures 9 and 10.

Strain	IC_{50} [µM]	95% Confidence Interval
BW25113 (wt)	0.032	0.022 to 0.047
ΔthiP	0.93	0.74 to 1.2
ΔthiQ	1.5	1.0 to 2.1
ΔtbpA	1.3	0.97 to 1.7
ΔthiI	0.027	0.018 to 0.039
ΔthiK	n. a.	n. a.
ΔsufS	0.025	0.018 to 0.036
ΔthiD	0.019	0.012 to 0.030
ΔthiM	0.016	0.010 to 0.026
ΔiscS	n. a.	n. a.
ΔthiH	0.028	0.021 to 0.037
ΔthiS	0.033	0.018 to 0.061
ΔthiF	0.009	0.0062 to 0.013
ΔthiE	0.071	0.045 to 0.11
ΔthiC	0.023	0.015 to 0.035

ΔthiG	0.0091	0.0054 to 0.015	

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References

Baba, T., T. Ara, et al. (2006). "Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection." Molecular systems biology **2**: 2006 0008.

Begley, T. P., D. M. Downs, et al. (1999). "Thiamin biosynthesis in prokaryotes." Archives of microbiology **171**(5): 293-300.

Blount, K. F. and R. R. Breaker (2006). "Riboswitches as antibacterial drug targets." Nature biotechnology **24**(12): 1558-1564.

Bocobza, S., A. Adato, et al. (2007). "Riboswitch-dependent gene regulation and its evolution in the plant kingdom." Genes & development **21**(22): 2874-2879.

Breaker, R. R. (2011). "Prospects for riboswitch discovery and analysis." Molecular cell **43**(6): 867-879.

Chen, L., E. Cressina, et al. (2012). "Probing riboswitch-ligand interactions using thiamine pyrophosphate analogues." Organic & biomolecular chemistry **10**(30): 5924-5931.

Deigan Warner, K. H., Philip; Weeks, Kevin M.; Smith, Alison G.; Abell, Chris; Ferré-D'Amaré, Adrian R. (2014). "Validating fragment-based drug discovery for biological RNAs: Lead fragments bind and remodel the TPP riboswitch specifically." Chemistry & biology.

Diederich, L., L. J. Rasmussen, et al. (1992). "New cloning vectors for integration in the lambda attachment site attB of the Escherichia coli chromosome." Plasmid **28**(1): 14-24.

Edwards, T. E. and A. R. Ferre-D'Amare (2006). "Crystal structures of the thi-box riboswitch bound to thiamine pyrophosphate analogs reveal adaptive RNA-small molecule recognition." Structure **14**(9): 1459-1468.

Erixon, K. M., C. L. Dabalos, et al. (2007). "Inhibition of pyruvate decarboxylase from Z. mobilis by novel analogues of thiamine pyrophosphate: investigating pyrophosphate mimics." Chem Commun (Camb)(9): 960-962.

Erixon, K. M., C. L. Dabalos, et al. (2008). "Synthesis and biological evaluation of pyrophosphate mimics of thiamine pyrophosphate based on a triazole scaffold." Organic & biomolecular chemistry **6**(19): 3561-3572.

Iwashima, A., Y. Wakabayashi, et al. (1976). "Formation of Pyrithiamine Pyrophosphate in Brain-Tissue." Journal of Biochemistry **79**(4): 845-847.

Kubodera, T., M. Watanabe, et al. (2003). "Thiamine-regulated gene expression of Aspergillus oryzae thiA requires splicing of the intron containing a riboswitch-like domain in the 5'-UTR." FEBS letters **555**(3): 516-520.

Lünse, C. E., A. Schüller, et al. (2014). "The promise of riboswitches as potential antibacterial drug targets." International journal of medical microbiology : IJMM **304**(1): 79-92.

Lutz, R. and H. Bujard (1997). "Independent and tight regulation of transcriptional units in Escherichia coli via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements." Nucleic acids research **25**(6): 1203-1210.

Mayer, G., M. S. Raddatz, et al. (2007). "RNA ligands that distinguish metabolite-induced conformations in the TPP riboswitch." Angewandte Chemie **46**(4): 557-560.

Miranda-Rios, J., M. Navarro, et al. (2001). "A conserved RNA structure (thi box) is involved in regulation of thiamin biosynthetic gene expression in bacteria." Proceedings of the National Academy of Sciences of the United States of America **98**(17): 9736-9741.

Morett, E., J. O. Korbel, et al. (2003). "Systematic discovery of analogous enzymes in thiamin biosynthesis." Nature biotechnology **21**(7): 790-795.

Rentmeister, A., G. Mayer, et al. (2007). "Conformational changes in the expression domain of the Escherichia coli thiM riboswitch." Nucleic acids research **35**(11): 3713-3722.

Rodionov, D. A., A. G. Vitreschak, et al. (2002). "Comparative genomics of thiamin biosynthesis in procaryotes. New genes and regulatory mechanisms." The Journal of biological chemistry **277**(50): 48949-48959.

Serganov, A., A. Polonskaia, et al. (2006). "Structural basis for gene regulation by a thiamine pyrophosphate-sensing riboswitch." Nature **441**(7097): 1167-1171.

Simons, R. W., F. Houman, et al. (1987). "Improved single and multicopy lac-based cloning vectors for protein and operon fusions." Gene **53**(1): 85-96.

Sudarsan, N., J. E. Barrick, et al. (2003). "Metabolite-binding RNA domains are present in the genes of eukaryotes." RNA **9**(6): 644-647.

Sudarsan, N., S. Cohen-Chalamish, et al. (2005). "Thiamine pyrophosphate riboswitches are targets for the antimicrobial compound pyrithiamine." Chemistry & biology **12**(12): 1325-1335.

Thore, S., M. Leibundgut, et al. (2006). "Structure of the eukaryotic thiamine pyrophosphate riboswitch with its regulatory ligand." Science **312**(5777): 1208-1211.

Webb, E., K. Claas, et al. (1998). "thiBPQ encodes an ABC transporter required for transport of thiamine and thiamine pyrophosphate in Salmonella typhimurium." The Journal of biological chemistry **273**(15): 8946-8950.

Winkler, W., A. Nahvi, et al. (2002). "Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression." Nature **419**(6910): 952-956.

Figure and Scheme Legends

Figure 1: (A) Secondary structure of the aptamer domain of the *E. coli thiM* riboswitch and the non-binding *thiM* riboswitch variant (*thiM*-Mu), whose alterations in P2 are shown in green (Mayer, Raddatz et al. 2007). (B) Schematic drawing of plasmid constructs and screening set-up employed for evaluating effects of thiamine analogs (compounds) in DH5αZ1 cells or Keio collection strains. When thiamine is added, it is taken up by the bacteria and converted to thiamine pyrophosphate, which then acts on the *thiM* riboswitch causing a repression of reporter gene expression. Levels of the reporter gene β-galactosidase are assayed by cell lysis and reaction with *ortho*-nitrophenyl β-galactoside (ONPG) (see methods for detailed procedure).

Figure 2: (A) Chemical structures of thiamine (T), pyrithiamine (PT) and compounds **1-6** that were assayed for influence on *thiM* riboswitch-dependent reporter gene expression and bacterial growth inhibition. (B) Relative β-galactosidase expression in the presence of either thiamine [20 µM] or compounds **1-6** and pyrithiamine (**PT**) [500 µM] in *E. coli* DH5αZ1 pRS414.2 *thiM* wt. Relative β-galactosidase expression in the presence of either thiamine [20 µM] or compounds **1-5** and pyrithiamine (**PT**) [500 µM] *E. coli* DH5αZ1 pRS414.2 *thiM*-Mu (C). As compound **6** did not show significant effects in primary screenings (B) it was not used for further investigations (C). Reporter gene activity is shown in relation to maximum β-galactosidase expression in DH5αZ1 bacteria (no addition of thiamine, black bars) and addition of 500µM thiamine yield maximal reporter gene repression (dark grey bars).

Scheme 1: Biosynthesis of TPP in *E. coli* is accomplished by the separate synthesis of the pyrimidine and thiazole moiety, which are finally coupled and phosphorylated to form TPP (Begley, Downs et al. 1999). The biosynthesis of the pyrimidine part of thiamine starts with an intermediate of the purine biosynthesis pathway, namely aminoimidazole ribotide (AIR). The thiamine biosynthesis protein ThiC whose exact function remains to be elucidated, converts AIR to hydroxymethylpyrimidine-phosphate (HMP-P), which is subsequently phosphorylated by the bifunctional HMP/ HMP-P kinase ThiD to yield hydroxymethyl-pyrimidine pyrophosphate (HMP-PP). The thiazole moiety of thiamine is derived from tyrosine, cysteine and 1-deoxy-D-xylulose phosphate (DXP). In a yet unresolved chain of reactions featuring thiF, thiS, thiG, thiH and thiI gene products, hydroxyethyl-thiazole phosphate (HET-P) is formed. HMP-PP and HET-P are joined by one enzymatic step mediated by the ThiE protein, followed by phosphorylation of the formed TMP by ThiL to create TPP. Three distinct kinases, ThiM, ThiD, and ThiK, are involved in the salvage of HET, HMP, and thiamine, respectively, from the culture medium. Thiamine, thiamine phosphate, and thiamine pyrophosphate are actively transported in enteric bacteria using the ABC transport system ThiBPQ (Webb, Claas et al. 1998). At present, no other distinct thiamine transporters, neither HET nor HMP transport systems, have been identified in bacteria (Rodionov, Vitreschak et al. 2002). Essential genes are colored red, gray genes are not validated in *E. coli*.

Figure 3: Effect of thiamine (A), triazolethiamine (B) or pyrithiamine (C) on reporter gene expression in BW25113 (black bars) and BW25113 strains with the indicated deletion. Minimal medium was supplemented with 500µM of thiamine (**T**), **TT** or **PT**. magenta bars: thiamine transport genes; green bars: phosphorylation enzymes; grey bars: genes involved in thiazole formation.

Figure 4: Chemical formulas of triazole thiamine analogs with phosphate mimicking groups (A). Relative β-galactosidase expression in the presence of thiamine or compound [500µM] in DH5αZ1 cells is shown (B).

Figure 5: Effect of thiamine, TT, PT and phosphate mimics **7** and **16** on β-galactosidase expression in *E. coli* strains *ΔthiK* (A), BW25113 (B) and *ΔthiP* (C). Minimal medium (refer to experimental procedures for details) was supplemented with 500µM of analyte. Compound **16** was dissolved to a stock solution of 100mM in 100% DMSO, assay controls also contained a final DMSO concentration of 1%.

Figure 1.TIF

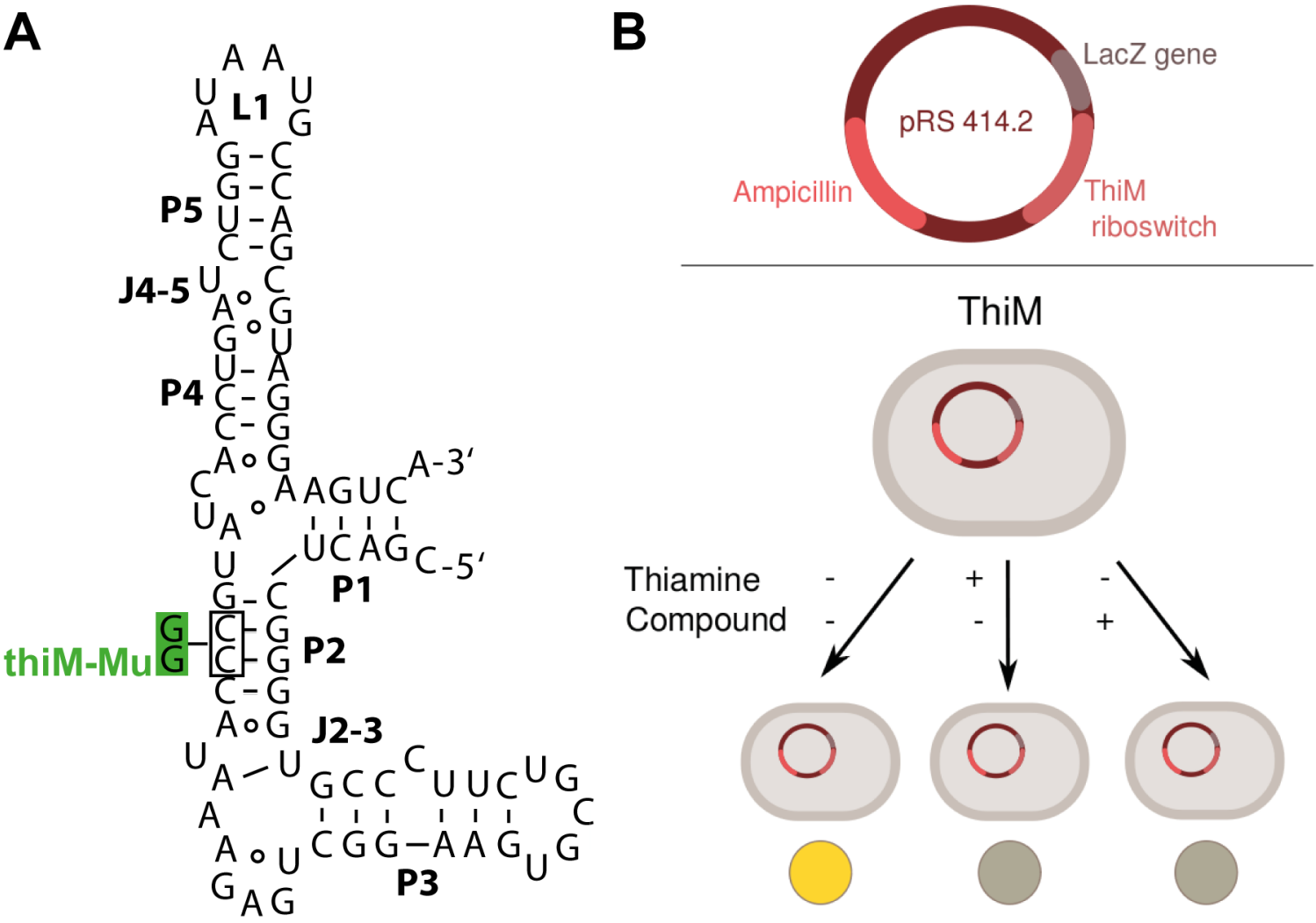
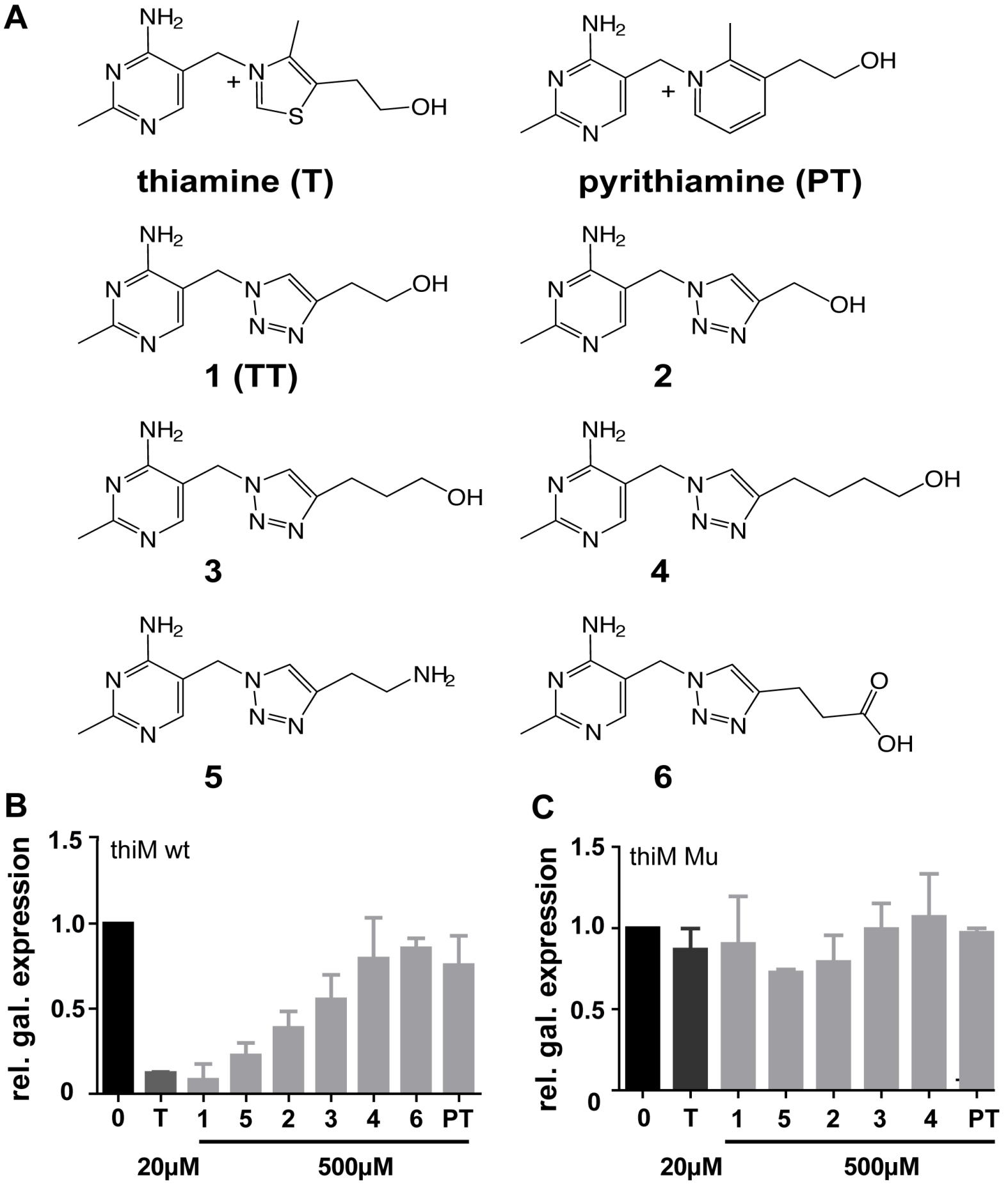

A
L1
P5
J4-5
P4
A-3′
AAGUC
UCAG
C-5′
P1
thiM-Mu
P2
J2-3
GCCC
UUC
CGG
AAG
P3
B
LacZ gene
pRS 414.2
Ampicillin
ThiM
riboswitch
ThiM
Thiamine
Compound
-
+
-
-
-
+

Figure 2.TIF


A
thiamine (T)
pyrithiamine (PT)
1 (TT)
2
3
4
5
6
B
thiM wt
rel. gal. expression
1.5
1.0
0.5
0
0 T 1 5 2 3 4 6 PT
20µM
500µM
C
thiM Mu
rel. gal. expression
1.5
1.0
0.5
0
0 T 1 5 2 3 4 PT
20µM
500µM

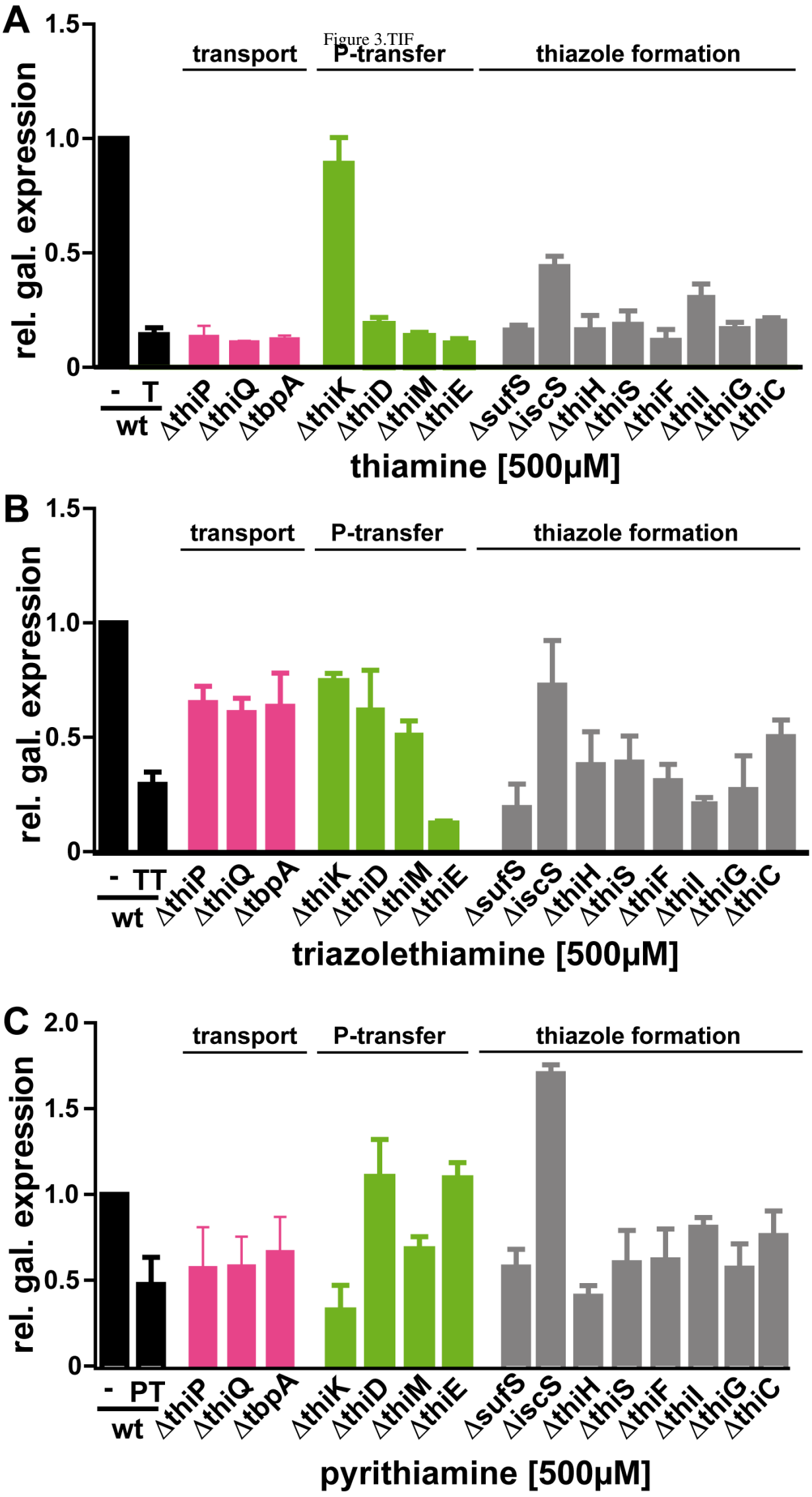
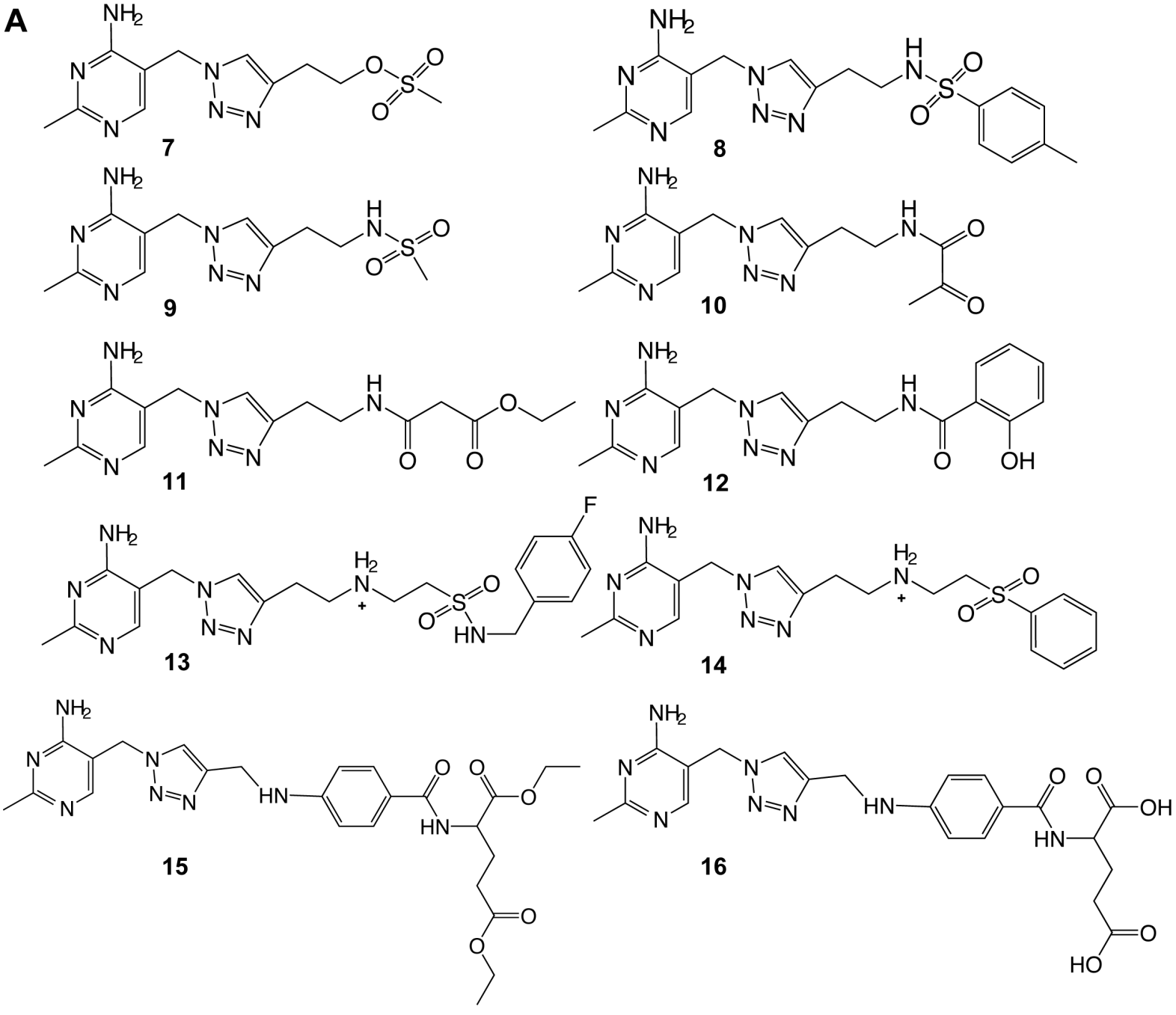


Figure 3.TIF
A
rel. gal. expression
1.5
1.0
0.5
0
transport
P-transfer
thiazole formation
- T
wt
ΔthiP
ΔthiQ
ΔtbpA
ΔthiK
ΔthiD
ΔthiM
ΔthiE
ΔsufS
ΔiscS
ΔthiH
ΔthiS
ΔthiF
ΔthiI
ΔthiG
ΔthiC
thiamine [500μM]
B
rel. gal. expression
1.5
1.0
0.5
0
transport
P-transfer
thiazole formation
- TT
wt
ΔthiP
ΔthiQ
ΔtbpA
ΔthiK
ΔthiD
ΔthiM
ΔthiE
ΔsufS
ΔiscS
ΔthiH
ΔthiS
ΔthiF
ΔthiI
ΔthiG
ΔthiC
triazolethiamine [500μM]
C
rel. gal. expression
2.0
1.5
1.0
0.5
0
transport
P-transfer
thiazole formation
- PT
wt
ΔthiP
ΔthiQ
ΔtbpA
ΔthiK
ΔthiD
ΔthiM
ΔthiE
ΔsufS
ΔiscS
ΔthiH
ΔthiS
ΔthiF
ΔthiI
ΔthiG
ΔthiC
pyrithiamine [500μM]

Figure 4.TIF


A
7
8
9
10
11
12
13
14
15
16

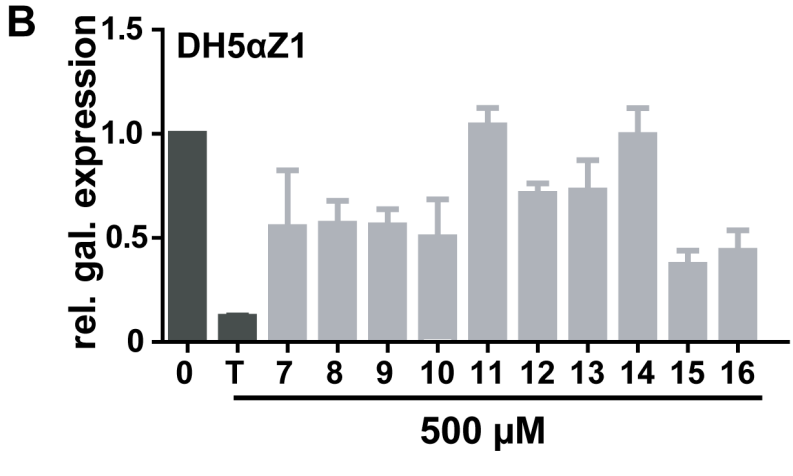
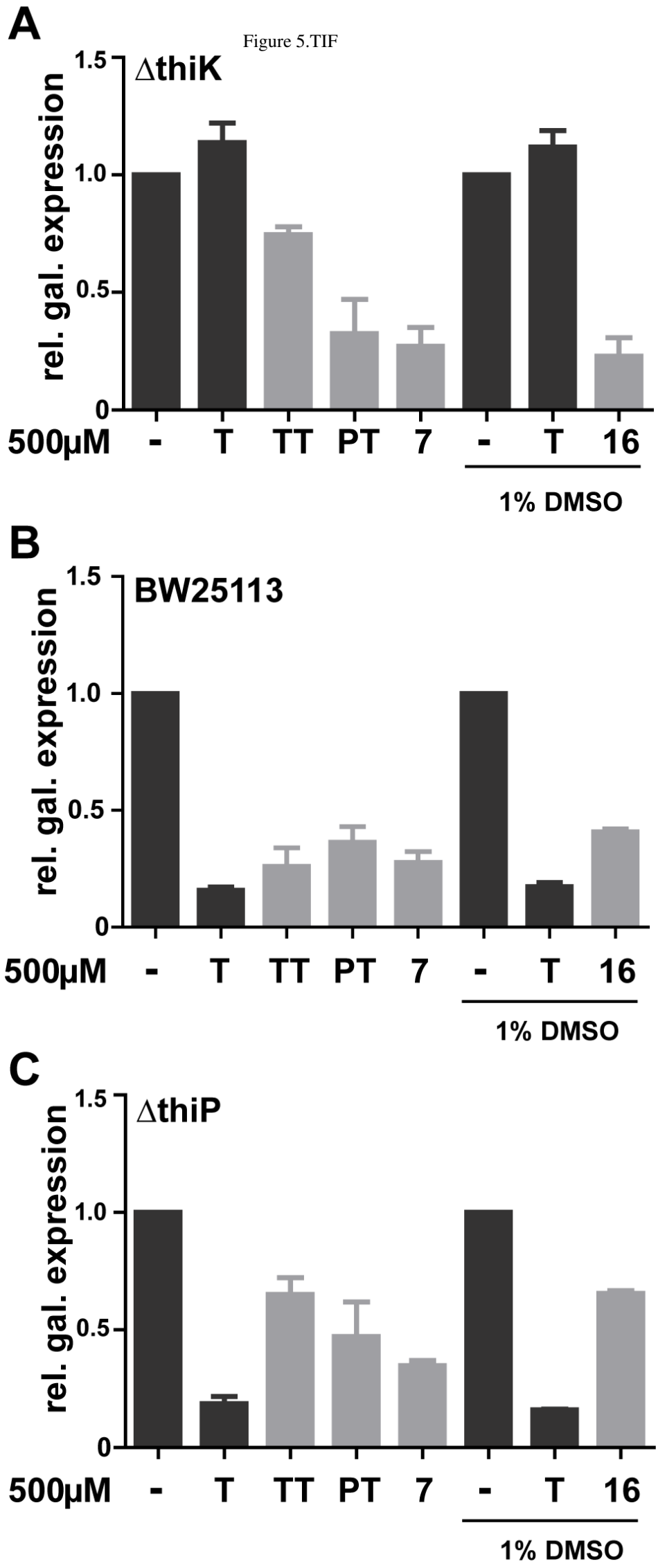

B
DH5αZ1
rel. gal. expression
1.5
1.0
0.5
0
0 T 7 8 9 10 11 12 13 14 15 16
500 μM

Figure 5.TIF


A
ΔthiK
rel. gal. expression
1.5
1.0
0.5
0
500μM - T TT PT 7 - T 16
1% DMSO
B
BW25113
rel. gal. expression
1.5
1.0
0.5
0
500μM - T TT PT 7 - T 16
1% DMSO
C
ΔthiP
rel. gal. expression
1.5
1.0
0.5
0
500μM - T TT PT 7 - T 16
1% DMSO